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13. ABSTRACT (Maximum 200 Words) We are currently investigating a putative tumor suppressor gene (TSG) located at chromosomal band 8p22 that is involved in breast cancer. We have proposed to apply the genetic suppressor element (GSE) approach to the identification of this TSG. Briefly, a library of short gene fragments will be introduced into a cell line which demonstrates suppression of clonogenicity in soft agar with the transfer of chromosome 8. Presumably, the 8p22 TSG is responsible for the suppression of clonogenicity, and the introduction of an "active" GSE from the library into the suppressed cells should inhibit the 8p22 TSG contained in the hybrid cells and allow reversion back to the parental phenotype, the ability to grow in soft agar. Any clones that form will be isolated and further evaluated, as a candidate for the TSG. Currently, several ESTs have been identified in the 8p22 interval using various methods. Construction of the GSE library consisting of these ESTs is currently underway. All preliminary optimization experiments for retroviral library delivery have been completed. Any positive GSE clones identified will be characterized and evaluated as candidate TSGs.				
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Breast cancer is generally characterized as a number of pathologically distinct entities that constitute the progression of lesions from hyperplasia, to carcinoma in situ, to invasive carcinoma, finally resulting in disease metastasis (1). Tumor suppressor genes (TSG) have been known to play an important role in the initiation and progression of human cancers, and there exists a large body of data that suggests the existence of a TSG on the short arm of chromosome 8 (8p). 8p is of particular interest in breast cancer because it is a site of frequent allelic loss in breast tumors, and it has been shown that 8p loss is one of the most common events in breast cancer (2). However, 8p loss is seen in a wide range of epithelial carcinomas (3-7), and is thought to be non tissue-specific. Several regions of 8p have been identified by LOH as putative TSG sites, including 8p22, where a homozygous deletion was reported in prostate cancer (8). Since homozygous deletions are regarded as the most sensitive indicator of TSG presence, we have chosen to concentrate our efforts in this region. Furthermore, others in our lab have shown functional evidence that chromosome 8 harbors tumor suppressor activity through monochromosome 8 transfer into cell lines derived from breast tumors, resulting in a decrease in tumorigenicity and clonogenicity in soft agar (unpublished). These matched cell line pairs offer an in vitro phenotype that is easily scorable and provides a model assay system. Using this model, we have proposed to use a functional negative selection approach to identify a tumor suppressor gene on 8p that is involved in breast carcinoma. In brief, we have proposed to construct a library of genetic suppressor elements (GSE), which consists of short gene fragments which encode inhibitory sense or antisense RNA (9-10), and introduce this library into our suppressed chromosome 8 containing breast cancer cell lines. Any active GSEs will inhibit the function of the putative TSG on 8p in these suppressed lines and as a result, "unsuppress" the cell clone, thus allowing colony formation in soft agar. Isolation, expansion, identification, and verification of the clones will follow. Finally, any genes identified will be characterized, and evaluated as a candidate TSG. Our progress is outlined below.

Objective 1: Creation of GSE library (Task 1)

During this past grant period, we have deviated slightly from our proposed statement of work. We had originally proposed to prepare a genetic suppressor element (GSE) library using bacterial clones from a physical map of 8p22 that we have constructed in our laboratory. However, since our original submission, significant progress has been made towards gene identification in the interval. Using specific cDNAs in place of genomic DNA for library construction offers several advantages. The resulting library has increased specificity while the complexity of the library is decreased. Thus, we have decided to construct a more specific library using ESTs that have been mapped to and around our interval in place of the bacterial contig.

In addition to our physical mapping effort, we have identified several ESTs that map to our region from the Human Transcript Map, by exon trapping, and analysis of the Radiation Hybrid database (11) (Table 2). We identified three ESTs by exon trapping; two ESTs were identified from the Human Transcript Map, and one additional EST was mapped to our region from the RHdb. One EST, D78237, that had been published by another group also mapped to 8p22 (12). Additional exons within the interval were

predicted by sequence analysis of genomic clones that have been mapped to the interval. Three genomic clones, AC008005, AF126483, and AC019292 have been localized to 8p22, and this has resulted in the identification of eight additional exons to be tested in our library.

We have expanded our investigation to include possible targets of the 8p22 TSG as well. Others in our laboratory performed differential gene expression studies using Genefilters to compare the expression patterns of the HT29 parental and chromosome 8 containing suppressed hybrid, HT29.8 cell lines. Since these cell lines differ genetically only by one copy of human chromosome 8, we can suggest that any significant differences in the expression patterns of the two cell lines can be attributed to the resultant actions of a gene on chromosome 8, presumably the 8p TSG. Four genes emerged from our GeneFilters studies as showing enhanced expression in suppressed HT-29.8 cells. Since the 8p22 TSG is thought to be non tissue-specific, we tested these genes for their expression pattern in the breast cancer hybrids, MDA-MB231.8-1 and ZR75.8-1, when compared to the parental cell line using semi-quantitative RT-PCR. The results are shown in Table 1 below.

Table 1: Gene Expression Analysis

Gene ID	HT29	MDA-MB231	ZR75
Hs. 96334	increase (1.5)	increase (1.4)	Decrease
Hs. 107056	increase (2.5)	increase (1.5)	Decrease
Hs. 17969	increase (1.6)	increase (1.75)	Decrease
Hs. 83883	increase (21.5)	increase (1.5)	increase (4.85)

"Increase" or "decrease" refers to the direction of change of expression level in the chromosome 8 containing hybrid cells when compared to the parental cells. The fold of increase is given in parentheses.

While all of the genes tested show some increase in the suppressed breast cancer hybrid cell lines, hs. 83883 showed the most compelling increase in both HT29.8 and ZR75.8-1 cells. Nevertheless, we will include all of these genes in our GSE library for evaluation in our model system.

Cloning of the GSE library in the retroviral vector pBABE, is currently underway. The list of genes and ESTs that we have identified thus far and are including in the library is given below in Table 2. In addition, we have already subcloned a GFP-expressing vector to serve as a positive control in our experiments, so that we may visually monitor the progress and efficiency of each step.

Table 2: List of ESTs to be included in GSE library

Sequence Name	Identification Source	Known Identity or Similarity
R80652	RHdb, Human Transcript Map	
R26196	RHdb	
WI-17604	Human Transcript Map	
20B08e1	Exon Trapping	Similar to Phosphatidylinositol-4-phosphate 5-kinase, type II
20B08e4	Exon Trapping	Similar to N-acetylgalactosaminyl

		transferase
20B08e5	Exon Trapping	
D78237	Exon Trapping (Published)	Similar to sperm surface protein PH-20
AF126283.1	Predicted from Genomic sequence	
AF126283.2	Predicted from Genomic sequence	Similar to phosphatase c
AF126283.3	Predicted from Genomic sequence	Similar to phosphatase c
AF126283.4	Predicted from Genomic sequence	
AF126283.5	Predicted from Genomic sequence	
AF126283.6	Predicted from Genomic sequence	
AF126283.7	Predicted from Genomic sequence	
AF126283.8	Predicted from Genomic sequence	
Hs. 107056	Genefilters	CED-6 gene
Hs. 17969	Genefilters	RNF11 gene
Hs. 96334	Genefilters	KIAA0663 gene product
Hs. 83883	Genefilters	PMEPA1 gene

Objective 2: Introduction of library into target cells (Task 1)

We have completed all preliminary studies to optimize the retroviral packaging system and soft agar cloning assay. Our ultimate goal, identification of biologically active GSEs, relies on the ability to perform highly efficient transfer of our library. To ensure a high level of transduction, it is necessary to optimize the system to fit our specific experimental conditions. Originally, we had proposed to use PT67 cells, which is an NIH-3T3 based packaging cell line. However, in our preliminary studies, we discovered that these cells do not have a very high transfection efficiency, and virus titers from these cells were not very impressive either. After extensive experimentation with a few commonly used cell lines, we decided on Phoenix A cells, which are 293-derived amphotropic packaging cells. Optimizing our transfections with a control vector containing an alkaline phosphatase reporter into the Phoenix A cells, we were able to achieve transfection efficiencies of approximately 40%. Furthermore, the transfected cells had an almost 10-fold increase in virus titer, 1×10^6 cfu/ml, over the PT67 cells.

We also performed parallel experiments using various transfection and infection methods to determine the most effective ones. We found that electroporation and CaPO_4 methods had much higher transfection rates over liposome mediated transfection. However, electroporation showed inconsistent results between experiments. CaPO_4 transfection gave the highest levels of transfectants, while providing consistent results throughout our experiments, and thus is the method of choice.

There are several suggested techniques to increase virus titer and infection efficiencies. Infection rates can be affected by the duration of incubation with virus, superinfection, concentration of virus, centrifugation, and addition of other agents such as polybrene. We experimented with several of these components to achieve optimal infections. We found that infection efficiency could be greatly enhanced by treatment with polybrene, and by performing up to three superinfections. The infections seemed also to be enhanced by centrifugation of the virus containing supernatant on to the target cells,

however, this effect cannot be explained. Our optimized infection efficiencies in the target cell lines ranged from 40-50%.

To determine the optimal number of cells to be plated, we performed soft agar colony formation assays. It is important to optimize the clonogenic efficiency such that enough cells are plated that all GSEs are represented, but few enough that the background is low and the clones can be easily scored. We determined the cloning efficiency of the MDA-MB231 and ZR75 cells to be approximately 1%. To determine the number of cells that must be plated, the library complexity must be taken into account. Our interval consists of about 20 ESTs. These ESTs represent approximately 20 kb of sequence, based on the fact that the average size of an insert in an IMAGE clone is about 1 kb. In order to obtain 20x coverage in the library (to assure representation of all sequences) for the 20 kb of total cDNA sequence in 200 bp fragments, we need to have about 2,000 independent clones. We plan to generate an excess of 20,000 clones to detect at least 10 active GSEs (if the library contains only one active GSE fragment). This number must be increased by a factor of 200 to account for the cloning efficiency of the parental MDA-MB231 and ZR75 cells, which is about 0.5%, to result in a total of 4×10^6 cells. This number should be doubled, to factor in the infection efficiencies of approximately 50%, yielding 8×10^6 cells to be plated. We determined the optimal plating density to be 1×10^5 cells per plate, which produces a scorable number of colonies in the parental cell lines.

Experimental conditions have thus been worked out for introduction of the GSE library in to the target cells. Further progress towards this end is awaiting completion of the library. The changes that we discussed above in construction of the library from the original proposal has slightly delayed its completion, however, this will be accomplished soon, and we will proceed as previously proposed

Objective 3: Characterization of GSEs (Task 2)

As a result of our deviation from the original proposal in Task 1, we have already accomplished part of Task 2 as outlined in the statement of work. We have already obtained the full-length cDNA sequence for several of these candidates, and are attempting to accomplish this for all of them. In the event that our GSE library does not yield any active GSEs, we will be equipped to test each EST individually. In addition, all the candidates which are being included in our GSE library have been tested, and are periodically rechecked for match of identity or homology with any known sequences or previously identified transcripts. This information is given in Table 2 above.

Characterization of identified GSEs is the proposed task for the current and upcoming funding periods, and this will be addressed in the following report.

Training

During this past funding period, all necessary coursework towards the doctoral degree has been completed. This has included courses in Biochemistry, Molecular Pathology, and Bioinformatics (audit). These classes, along with those that were previously taken

have provided a strong background and foundation for investigation in the laboratory. This training has been supplemented by participation in regular journal clubs, seminars, lab meetings, and workshops. In addition, attending the national meeting for the American Society of Human Genetics provided invaluable learning experiences that are necessary for becoming a successful researcher.

Key Accomplishments

- Gene/EST identification within the 8p22 TSG interval.
- Gene identification of possible 8p TSG functional targets.
- Optimization of retroviral packaging system and soft agar cloning assay with control experiments.
- Cloning of a GFP-containing vector construct as a control to determine transfection and infection efficiencies.
- Completion of all coursework necessary for obtaining the doctoral degree, including biochemistry, molecular pathology, and bioinformatics (audit)
- Attending a national meeting of the American Society of Human Genetics.

Reportable Outcomes

Arbieva, ZH, Edassery SL, **Banerjee K**, Kim SY, Horrigan SK, Westbrook CA. The 8p22 tumor suppressor gene site: Identification of novel expressed sequences. Am. J. Human Genetics 65, (suppl): A117, 1999.

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